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Metabolic modelling and energy parameter estimation of *Tetradesmus obliquus*

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ABSTRACT

We developed a metabolic network describing the primary metabolism of *Tetradesmus obliquus* aimed to get a better understanding of metabolism to improve industrial production. The network includes 351 reactions with 183 metabolites distributed over 4 compartments: cytosol, chloroplast, mitochondria, and extracellular space. The energy requirements for biomass assembly and maintenance (K_x and m_{ATP}, respectively) were experimentally determined from batch cultures and included in the model. The determined values were 121.02 $mmol_{ATP} \cdot g_{DW}^{-1}$ for K_x and 0.66 $mmol_{ATP} \cdot g_{DW}^{-1} \cdot h^{-1}$ for the m_{ATP}. The maintenance value found for *T. obliquus* is, to our knowledge, one of the lowest reported in literature for microalgae. This low value is also in agreement with the photon maintenance requirement found experimentally for *T. obliquus* (1.18 $mmol_{ph} \cdot g_{DW}^{-1} \cdot h^{-1}$). Finally, the theoretical maximum yields based on the model for biomass, triacylglycerides (TAG), and starch yield on light were calculated to be 1.15 $g \cdot mol_{ph}^{-1}$, 1.05 $g_{TAG} \cdot mol_{ph}^{-1}$, and 2.69 $g_{starch} \cdot mol_{ph}^{-1}$.

1. Introduction

Microalgae are promising sustainable cell factories for the production of food, feed, nutraceuticals, chemicals, and fuels due to their photosynthetic ability and product diversity [1,2]. The oleaginous microalgae *Tetradesmus obliquus* (formerly known as *Scenedesmus obliquus* [3]) is an industrially relevant strain that can accumulate high amounts of lipids [4–7]. *T. obliquus* reached a maximum triacylglyceride (TAG) yield on light of 0.14 $g_{TAG} \cdot mol_{ph}^{-1}$ and a final TAG content of 0.45 $g \cdot g_{DW}^{-1}$ under batch nitrogen starvation conditions [8].

At this moment the bulk production of microalgal products is not economically feasible, which is partly due to cultivation costs [9]. The production costs can be lowered by increasing the yield based on light for a desired product. Strategies to accomplish this involve the steering of algal metabolism towards the product of choice and increasing the efficiency with which the light is used. However, finding the right pathways and enzymes to target in order to reach this is difficult due to a lack of understanding of algal metabolism. Genome-scale metabolic models are a powerful tool for obtaining a better understanding of metabolism and are used for several applications, such as finding targets for metabolic engineering or elucidating regulatory mechanisms in a metabolic network [10].

Several metabolic models can be found in literature for eukaryotic microalgae. A first reconstruction of the metabolic network of *Chlorella sorokiniana* contained 67 reactions and 61 metabolites, with a low level of compartmentalisation as little was known regarding localization [11]. With the sequence and annotation of the genome of the model microalgae *Chlamydomonas reinhardtii* [12] a large amount of information about the metabolism became available and metabolic models were developed [13–19]. These models had a higher level of compartmentalisation, as well as a higher number of reactions and metabolites.

The problem with the genome-scale models is that both the annotation and prediction in which compartment a protein ends up, is poor [20]. As an alternative to the genome scale models, core models are developed containing the primary metabolism [13,18,21,22]. These models differ in their representation of internal metabolism, as all the reactions that are annotated in the genome are present in the genome-

Abbreviations: K_x, growth associated maintenance or energy requirement for biomass assembly; m_{ATP}, non-growth associated maintenance or maintenance energy; mol_{ph}, mol of photons; DW, dry weight; OD_{750}, optical density at 750 nm; TAG, triacylglycerides; FBA, Flux Balance Analysis; q_{ATP}, net specific ATP production rate; μ, specific growth rate calculated at different time points; C_x, biomass concentration

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scale models. However, both models can correctly make predictions, as shown for *E. coli* with a genome scale metabolic model [23] as well as a core model [21]. Additionally, the primary metabolism is usually well annotated and information on localisation of metabolic pathways can be obtained from literature.

As for genome-scale models, the amount of energy needed for making biomass (growth-associated maintenance, K_x) and for maintenance (non-growth-associated maintenance, m_{ATP}) are two important unknown parameters that must be determined experimentally. A common strategy to estimate the energy parameters is using a chemostat set-up. However, if for both systems the maximum amount of light per cell is the same, the batch approach in principle covers the same range of growth rates as the chemostat approach. For algae growing under light-limited batch cultures, the available light per cell slowly decreases due to the growth of the algae themselves. Thus, the batch approach allows going slowly through a whole range of different specific growth rates throughout the cultivation, whereas in a chemostat usually a limited number of growth rates is tested.

In this paper we developed a core model for *T. obliquus*. We verified whether the genes responsible for the reactions in the model are present in the genome as published by Carreres et al. [24]. Then we determined experimentally the energy parameters for maintenance and biomass formation from batch cultivations and included them in the model. Finally, the model was used to predict the theoretical yields of biomass, starch and triacylglycerides (TAG) on light.

2. Materials and methods

2.1. Model development and Flux Balance Analysis (FBA)

An organism's metabolism can be described by a set of stoichiometric reactions [25]. We constructed a de novo metabolic network for *Tetradesmus obliquus* where the reactions were included in either chloroplast, cytosol or mitochondria based on information from text books and literature [14,26–29]. Afterwards, the transport steps between the compartments were added. Finally, we checked whether the genes responsible for the included reactions were present in the genome reported by Carreres et al. [24].

After the biochemical network was developed, the intracellular fluxes of the network were studied using Flux Balance Analysis (FBA). An objective function was set and the solution space was narrowed down by including constraints. Such constraints included the quasi steady-state constrain where the accumulation of intermediate metabolites as well as the depletion is assumed to be zero (this is, no dynamics for the intracellular metabolites), setting boundaries for measured reactions, and restricting the directionality of reactions based on thermodynamics. This is mathematically represented as:

Objective function:

$$\max(c \cdot v)$$

Constraints:

$$S \cdot v = 0$$

$$LB \leq v \leq UB$$

where S is the stoichiometric matrix S (where S_{ij} contains the stoichiometric coefficient of compound i in reaction j), c is a row vector which defines the objective function, and LB and UB are the lower and upper boundaries for the reaction rates of the reaction vector v. To estimate the energy parameters, the ATP production was used as an objective function and the specific growth rates calculated on different time points (Section 2.5) and the light supply rate (Section 2.6) were measured and used to constrain the solution space. The network contained several circulations and therefore a unique solution for the flux distribution cannot be calculated. Therefore, we used the geometric mean to single out a unique solution. The geometric mean represents a unique solution in the solution space, which minimizes the total flux in the cell, while satisfying the objective function, removing thermodynamically infeasible cycles and imposed constraints [30].

In silico simulations were calculated using the COBRA Toolbox [31,32] with the 'glpk' solver in MATLAB R2015b version 8.6 (The MathWorks Inc., USA).

2.2. Energy parameters

The growth associated maintenance (K_x) and the non-growth associated maintenance (m_{ATP}) parameters were calculated from experimental data applied to the model. For this calculation, the ATP balance is written as:

$$\sum s_i^{ATP} \cdot v_i - K_x \cdot \mu - m_{ATP} = 0 \quad (1)$$

The first term in the equation represents the net production of ATP in the network excluding ATP used for growth and maintenance. Here s_i^{ATP} is the stoichiometric coefficient of ATP in reaction i and v_i is the flux through reaction i. ATP production from oxidative phosphorylation and the light reactions are included in this term. The stoichiometry for the oxidation of NADH and $FADH_2$ in the oxidative phosphorylation is defined based on the P/O ratio (ratio of ATP synthesized per 1/2 mol of O_2 reduced to water). A P/O ratio of 2.5 and 1.5 is generally accepted for NADH and $FADH_2$, respectively [18], and was used for the model. With respect to ATP generation from light, the two most important processes that result in ATP and/or NADPH production were considered. During the linear electron transport (LET), according to the Z-scheme of photosynthesis, 3 ATP and 2 NADPH are produced for every 8 photons absorbed. Additionally, the cyclic electron transport (CET) yields 1 ATP per 2 photons absorbed [33]. Optimal photosynthetic efficiency of 8 photons for 3 ATP and 2 NADPH is in practice never achieved, possibly because always a certain minimal amount of CET occurs. Therefore a minimum requirement of 10 photons is assumed for LET to produce 3 ATP and 2 NADPH [33]. K_x represents the ATP requirement for the formation of biomass from biopolymers (in $mmol_{ATP} \cdot g_{DW}^{-1}$). This constant includes the processes that use ATP and are not defined as reactions in the network (e.g. the assembly of biopolymers like protein and lipids into functional cells). μ stands for the specific growth rate calculated on different time points (h^{-1}). The final term m_{ATP} (in $mmol_{ATP} \cdot g_{DW}^{-1} \cdot h^{-1}$) refers to the maintenance requirements of the cell, which vary depending on the species and culture conditions.

With the P/O ratio and the stoichiometry of the light reactions known, the first term can be calculated if the flux distribution is known. This term represents the net specific ATP production rate (q_{ATP}) in the network. Then Eq. (1) can be written as:

$$q_{ATP} = K_x \cdot \mu + m_{ATP} \quad (2)$$

The parameters K_x and m_{ATP} can now be experimentally estimated by plotting the simulated q_{ATP} against the specific growth rates calculated on different time points (μ). Here the slope is K_x and the intercept m_{ATP}. Alternatively, m_{ATP} can also be directly calculated from the q_{ATP} during the stationary phase ($\mu = 0$) of the batch culture.

To estimate K_x and m_{ATP}, a series of batch experiments were carried out under nutrient replete conditions and at low light intensity. Samples were taken at different time points of the batch cultivations to determine a wide range of specific growth rates. The maximum ATP production rate (q_{ATP}) was calculated using FBA. For each growth rate a separate biomass equation was used that matched the measured biomass composition at that specific growth rate.

2.3. Strains, pre-culture conditions and cultivation medium

Wild-type *Tetradesmus obliquus* UTEX 393 was obtained from the Culture Collection of Algae, University of Texas. Pre-cultures were